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OBSERVATIONS ON INDOL PRODUCTION BY BACTERIA OF THE COLON-TYPHOID GROUP.*

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While the production of indol in a peptone solution is extensively used as a test for certain groups of bacteria, most authors are rather skeptical as to its real classificatory value. Many variable results have been obtained by different workers, as a result of which the test has been greatly discredited. Nevertheless it is still used in most laboratories.

Recently Zipfel¹ suggested the use of tryptophan, the mother substance of indol (indol-a-amino-propionic acid), for testing the power of bacteria to produce indol. In a series of studies on the behavior of a large number of bacteria of different groups toward that substance, he obtained strikingly constant results. The organisms of the same group either all do or do not produce indol and generally in 24 hours. Seidelin,² however, showed that while bacteria were capable of producing indol from tryptophan, at no time during his tests could he demonstrate the presence of tryptophan in a peptone water culture of an indol-producing organism. Indol is produced from peptone and tryptophan, therefore, apparently by two distinct processes, and while the latter may be a useful test, it cannot take the place of the peptone test. Besides, tryptophan is so difficult either to prepare or obtain that its extensive use is out of the question at present.

In the present state of bacteriological technic, many reasons other than the variability of the reaction may be found to explain the inconstancy of a certain test. The technic may differ in several respects. Very often different methods are employed in preparing the peptone solution. Some use distilled water, others tap water, and still others add salt either to the distilled or to the tap

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¹ *Centrabl. f. Bakteriöl.*, I, *Orig.*, 1912, 64, p. 65; *ibid.*, 67, p. 572.

² *Jour. Hyg.*, 1911, 11, p. 503.

water solution. While these variations will not affect the actual production of indol they may affect the time in which sufficient indol is produced to respond to the test. Another factor is the mode of inoculation of the medium. The age and amount of the culture inoculated will, of course, influence the course of indol production. Finally the time of incubation varies widely; some employ 4, others 6, and still others 10 days for incubation. It is evident that the above factors when combined may have a decided influence on the recorded result.

The method used for making the test may also constitute a very serious source of error. In America the Salkowski test ($\text{H}_2\text{SO}_4 + \text{KNO}_2$) is widely used and recommended by the Committee on Standard Methods (1912) as a test for indol. Böhme (1905)¹ and Marshall (1907)² after a series of comparative tests concluded that the Ehrlich method (paradimethylamino-benzaldehyde+HCl) is more sensitive and gives more constant results. MacConkey (1909)³ claims that while the Salkowski test does give variable results, the Ehrlich test rarely varies.

While conducting a series of tests on certain members of the colon-typhoid group, I thought it desirable to ascertain the constancy of this reaction when the factors enumerated above are carefully controlled. The test was performed on the same series of organisms on three different occasions, using different incubation periods but employing both the Salkowski and the Ehrlich tests. In this way the constancy of the organism, the importance of the incubation period, and the relative value of the two tests were determined.

The technic employed was in brief as follows: a peptone solution consisting of H_2O (distilled), 1000 c.c.; peptone (Witte's), 10.0 gm.; K_2HPO_4 , 0.2 gm.; NaCl , 5.0 gm. was made up, filtered through filter paper, tubed, 10 c.c. to a tube, and autoclaved. This medium gave excellent growth.

Each culture to be tested was inoculated into a tube of this peptone broth and incubated for 24 hours. The 24-hour culture was then used for the inoculation of the peptone broth to be tested. One cubic centimeter of the peptone culture was inoculated into each of 5 tubes of broth by means of a sterile pipette, and these were incubated at 37°C . Periods of 2, 4, and 6 days respectively were employed for incubation, the tests being performed at approximately monthly intervals. In all of these cases the Ehrlich method was used.

¹ *Centralbl. f. Bakteriolog.*, I, Orig., 1905, 40, p. 129.

² *Jour. Hyg.*, 1907, 7, p. 581.

³ *Jour. Hyg.*, 1909, 9, p. 86

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For the determination of the relative value of the two tests, duplicate peptone broth tubes of each culture were incubated for 4 days. The contents of each tube were then divided into two parts, and subjected to the Ehrlich and Salkowski tests respectively. The same tube was thus tested by both methods. The rest of the technic was the same as above.

The Ehrlich test was made by adding 1 c.c. of a 2 per cent solution of the aldehyde in 95 per cent alcohol and then adding concentrated HCl drop by drop until a red zone appeared between the alcohol and peptone layers. Not more than 0.5 c.c. of acid is necessary. On standing for about 15 minutes the red zone deepens and forms a wider ring. Each tube was then shaken up with chloroform and when it dissolved the red color the test was considered positive. By using approximately the same amount of chloroform for each tube, an idea of the relative amount of indol formed can be obtained from the intensity of the color.

The Salkowski test was performed in the usual way by adding first 1 c.c. of 10 per cent H_2SO_4 and then slowly 1 c.c. of 0.01 per cent solution of potassium nitrite. A pink to red ring is thus formed which deepens on standing. In a few cases the whole tube reddened almost instantly, but on shaking with chloroform none of the color went into solution. This phenomenon will be referred to later.

In all, 75 cultures were tested, falling roughly into the following groups:

<i>B. communis</i>	13	<i>B. communior</i>	13
<i>B. aerogenes</i>	20	<i>B. cloacae</i>	9
<i>B. acidi-lactici</i>			
<i>B. proteus</i>	5	<i>Paratyphi</i> group	10

The results obtained are summarized in the tables below:

TABLE 1.
EHRlich METHOD.

GROUP	NO. OF CULTURES TESTED	INCUBATION PERIOD		
		2 Days	4 Days	6 Days
		No. of Positive Results	No. of Positive Results	No. of Positive Results
Communior	13	10	10	10
Communis	13	12	12	13
Aerogenes-lactici	20	6	8	8
Cloacae	9	0	0	0
Proteus	5	3	3	3
Paratyphi	10	0	0	0

Table 1 points out the constancy with which the organisms react when the test for indol is carried out according to the Ehrlich method. In the communior group the same 10 organisms were repeatedly positive while 3 were negative. All of the communis

organisms were repeatedly positive and in all duplicate tubes. Twenty-three out of 26 colon organisms, or 90 per cent, were thus repeatedly positive. This is all that can be expected and the colon bacillus is justly called indol positive.

Only 8 out of 20, or 40 per cent, of the aerogenes organisms are indol positive. With these weaker indol-producers 2 days' incubation is not sufficient for the test. On the whole the 4-day period is satisfactory.

All the cloacae and paratyphi are repeatedly negative, while the proteus cultures divide into two groups—one indol positive, the other indol negative.

In all cases all the tubes used for each organism reacted alike.

TABLE 2.
INCUBATION PERIOD—4 DAYS.

GROUP	NO. OF CULTURES TESTED	POSITIVE RESULTS OBTAINED WITH	
		Ehrlich Test	Salkowski Test
Communior	13	10	10
Communis	13	12	11
Aerogenes-lactici	20	8	8
Cloacae	9	0	2
Proteus	5	3	5
Paratyphi	10	0	1

Table 2 gives a comparison between the two methods. The Salkowski results are higher as was also noted by Marshall (1907).¹ Besides this there were five instances in which the Salkowski test gave positive results in one tube and negative in the other. These tests were counted positive for convenience, because both of the Ehrlich tubes of the same strain were positive. These aberrant results illustrate one of the possibilities of error. I am inclined to think that the negative Salkowski tests in this case were perhaps due to a rapid oxidation of the red coloring matter.

The high results obtained with the Salkowski test are attributable to the formation of a red color which apparently is not due to indol. If the test is carefully performed this reddening can be distinguished by its rapid diffusion throughout the tube from the

¹ *Loc. cit.*

red ring obtained with indol. This reaction was obtained in all those strains in which the Erhlich test was negative and the Salkowski positive. A similar reaction was obtained with a non-indol producing spore-former. In none of these cases was the color soluble in chloroform. Here then we evidently have a substance which gives a similar tho not the same reaction as indol and which can easily be confused with it. The results indicate that the Ehrlich test is to be preferred to the Salkowski.

An interesting phenomenon pointed out by Seidelin and Lewis (1911)¹ was also observed by me in connection with the Ehrlich test. This consists in the formation of a purple to bluish color which is insoluble in chloroform. Lewis claims that three distinct reactions are obtained: (1) soluble+insoluble red; (2) soluble red+purple or blue; (3) no soluble red, but insoluble blue. The soluble red observed by Lewis is, of course, the indol red. I have not met with any insoluble red pigment in my tests with the Ehrlich method. The purple color apparently is but a primary oxidation stage of the blue and both appear to be independent of indol production as indicated by the following observations:

1. If the tubes are shaken up with chloroform without the previous addition of persulfate the supernatant liquid is colorless. On standing for some time the liquid gradually assumes a purplish and eventually either blue or purple-blue color. This was observed in all cases. Often of two duplicate tubes one was purplish blue, the other blue.

2. The addition of a few drops of fuming HNO_3 or H_2O_2 (oxidizing agents) to the decanted supernatant liquid produced instantly the same changes of color observed on long standing.

3. The blue color reaction was obtained in uninoculated controls and also in a solution of peptone in distilled water treated with the aldehyde and hydrochloric acid. This shows that this reaction is independent of indol production. Since the aldehyde plus concentrated HCl alone does not give this reaction, it is evident that the blue coloration is due to the peptone. Whether it is specific for peptone or whether it is caused by one of the substances present in Witte's peptone mixtures still remains to be determined.

¹ *Loc. cit.*

CONCLUSIONS.

From the study presented above it appears:

1. That the indol reaction is sufficiently constant to be of diagnostic value.
2. That the Ehrlich test is constant and more reliable than the Salkowski. The test should be made on the fourth or sixth day and the tubes should always be shaken up with chloroform as a confirmatory test.
3. That the Salkowski test is unreliable because a red coloration is frequently obtained which is not due to indol, but to another substance and may be mistaken for it; and because the reaction in cultures which really produce indol is not constant.
4. That with the Ehrlich test *B. coli* is generally indol positive (+); *B. aerogenes* and *B. proteus*, variable (\pm); and *B. cloacae* and *B. paratyphi*, always negative (-).
5. That the blue color obtained in connection with the Ehrlich test is entirely independent of the indol test and is due to some substance present in the peptone.